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Title: Canine olfactory ensheathing cells from the olfactory mucosa can be engineered to produce active chondroitinase ABC.

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Abstract

A multitude of factors must be overcome following spinal cord injury (SCI) in order to achieve clinical improvement in patients. It is thought that by combining promising therapies these diverse factors could be combatted with the aim of producing an overall improvement in function. Chondroitin sulphate proteoglycans (CSPGs) present in the glial scar that forms following SCI present a significant block to axon regeneration. Digestion of CSPGs by chondroitinase ABC (ChABC) leads to axon regeneration, neuronal plasticity and functional improvement in preclinical models of SCI. However, the enzyme activity decays at body temperature within 24-72 hours, limiting the translational potential of ChABC as a therapy. Olfactory ensheathing cells (OECs) have shown huge promise as a cell transplant therapy in SCI. Their beneficial effects have been demonstrated in multiple small animal SCI models as well as in naturally occurring SCI in canine patients. In the present study, we have genetically modified canine OECs from the mucosa to constitutively produce enzymatically active ChABC. We have developed a lentiviral vector that can deliver a mammalian modified version of the ChABC gene to mammalian cells, including OECs. Enzyme production was quantified using the Morgan-Elson assay that detects the breakdown products of CSPG digestion in cell supernatants. We confirmed our findings by immunolabelling cell supernatant samples using Western blotting. OECs normal cell function was unaffected by genetic modification as demonstrated by normal microscopic morphology and the presence of the low affinity neurotrophin receptor ($p75^{NGF}$) following viral transduction. We have developed the means to allow production of active ChABC in combination with a promising cell transplant therapy for SCI repair.

Keywords
Chondroitinase ABC
Chondroitin sulphate proteoglycan
Olfactory ensheathing cells
Olfactory mucosa
Spinal cord injury
Gene therapy

Highlights

- Olfactory ensheathing cells from the mucosa can be genetically modified to secrete chondroitinase ABC.
- Lentiviral transduction of olfactory ensheathing cells did not change their microscopic morphology or cell marker expression ($p75^{NGF}$).
- Chondroitinase ABC production was confirmed using the Morgan-Elson assay and Western blot.

1. Introduction

Spinal cord injury (SCI) initiates a cascade of biochemical events leading to the formation of a glial scar (1, 2). The primary function of the glial scar is to restore the blood-spinal cord barrier to prevent an overwhelming inflammatory response following SCI (3, 4). However, injured axons are unable to regenerate across the site of injury in the central nervous system because of the presence of an array of inhibitory cues present within the scar (For a review, see 5, 6), in particular chondroitin sulphate proteoglycans (CSPGs) (7-10). Digestion of CSPGs with the bacterial enzyme chondroitinase ABC (ChABC), following local delivery to the spinal cord, has led to axon regeneration, plastic neuronal rearrangements and functional recovery following section or crush injury in laboratory animal SCI models (11-21). Encouragingly, these findings have also been found using clinically relevant contusive injury rodent models (22, 23) and large animal models such as cats and squirrel monkeys (19, 24, 25). Despite these advances, a major limitation for the use of ChABC in humans is the rapid decay of enzymatic activity at body temperature, within 24 to 72 hours (26). Therefore, serial intraspinal injections or infusion of fresh enzyme is required for efficacy. This represents a safety issue for its clinical application that needs to be addressed.

A modified variant of the bacterial *ChABC* gene, capable of producing active enzyme from mammalian cells, has recently been developed (27). This sequence has been incorporated into both adeno-associated viral vectors and lentiviral vectors as a delivery system of the modified *ChABC* gene into mammalian cells (22, 23, 28, 29). Lentiviral vectors transduce host cells efficiently and this results in long-term expression of the incorporated transgenes. They also are less immunogenic than other viral delivery systems such as adenoviral-based vectors (30, 31). *In vivo* studies testing viral transduction of cells within the spinal cord following intraparenchymal injection of a lentiviral vector containing the mammalian form of the *ChABC* gene resulted in widespread CSPGs digestion over several spinal segments and this persisted for at least 2 months (22, 23).

Abbreviations
ChABC, chondroitinase ABC; CSPGs, chondroitin sulphate proteoglycans; MOI, multiplicity of infection; NgR ecto, Nogo receptor ectodomain; OECs, olfactory ensheathing cells; OMCs, olfactory mucosa cells; $p75^{NGF}$, anti-nerve growth factor receptor; PTPσ, protein tyrosine phosphatase σ; SCI, spinal cord injury; VSVG, vesicular stomatitis virus envelope glycoprotein.

Here, we have taken the approach of transducing a population of cells *in vitro* to express the mammalian-modified form of ChABC. Our aim is to develop a new therapy that combines the beneficial effects of a cell transplant while allowing sustained delivery of ChABC into the site of SCI. It is generally agreed that combining therapies for SCI is likely to be more efficient than a single therapy to repair complex lesions affecting patients (32-34). ChABC is likely to form an essential component of any combination therapy because of its pleotrophic actions that compliment most other SCI therapies. It promotes plasticity, is neuroprotective and also has an anti-inflammatory action by promoting the polarization of macrophages to the anti-inflammatory M2 type (23).

We have chosen to work with olfactory ensheathing cells (OECs), a cell type that has been extensively studied for SCI repair. When transplanted into a damaged region of the central nervous system OECs can remyelinate, guide and support axons (35, 36). They also mediate their effects through the provision of growth factors that encourage plasticity, stimulate angiogenesis, interact with the glial scar and are involved in immune modulation (For a review, see 37, 38). Their beneficial effect on locomotion has been demonstrated in rodent models of SCI (39-43) and in companion dogs (44). Further, combining spinal cord injection of OECs and ChABC has a greater effect on locomotion and bladder voiding than either therapy alone (45, 46).

In the following sections, we present how canine OECs obtained from the olfactory mucosa (i.e. olfactory mucosa cells - OMCs) of companion dogs can be transduced in culture with a lentiviral vector to express a functional mammalian-modified form of ChABC.

2. Materials and Methods

2.1 Lentiviral production

The *proteus vulgaris ChABC* gene was previously modified to produce a mammalian-compatible *ChABC* gene by removing five cryptic N-glycosylation sites, which enabled its efficient and functional expression by mammalian cells (27). The mammalian *ChABC* gene was cloned into a lentiviral vector backbone (third-generation transfer vector pRRL; (47) to generate *pRRL-CMV-ChABC-SFFV-GFP* (see LV-ChABC construct in Figure 1). Large scale DNA purification of the lentiviral transfer plasmids and packaging plasmids were prepared by cesium chloride double ultracentrifugation followed by standard DNA precipitation. Lentiviral vectors pseudo typed with the vesicular stomatitis virus envelope glycoprotein (VSVG) coat were produced using the four plasmid transient transfection protocol as previously described (48). Briefly, human embryonic kidney cell (HEK293T) cultures were co-transfected with the lentiviral transfer plasmids containing the genes of interest (*ChABC* and / or *GFP*), and pMDLg/pRRE gag/pol, pMD2-VSVG and pRSV-Rev using lipofectamine mediated transfection. The cell supernatant containing viruses was harvested 24 and 48 hours post-transfection. Following filtration through a 0.45 µm Nalgene filter unit (Fisher Scientific, Loughborough, UK) the supernatant was concentrated 2000-fold by ultracentrifugation and the resultant viral pellet was re-suspended in TSSM (20 mM tromethamine, 100 mM NaCl, 10 mg/mL sucrose and 10 mg/mL mannitol) buffer. Viral vector titres were determined by flow cytometry (FACScalibur flow cytometer, BD Biosciences, New Jersey).

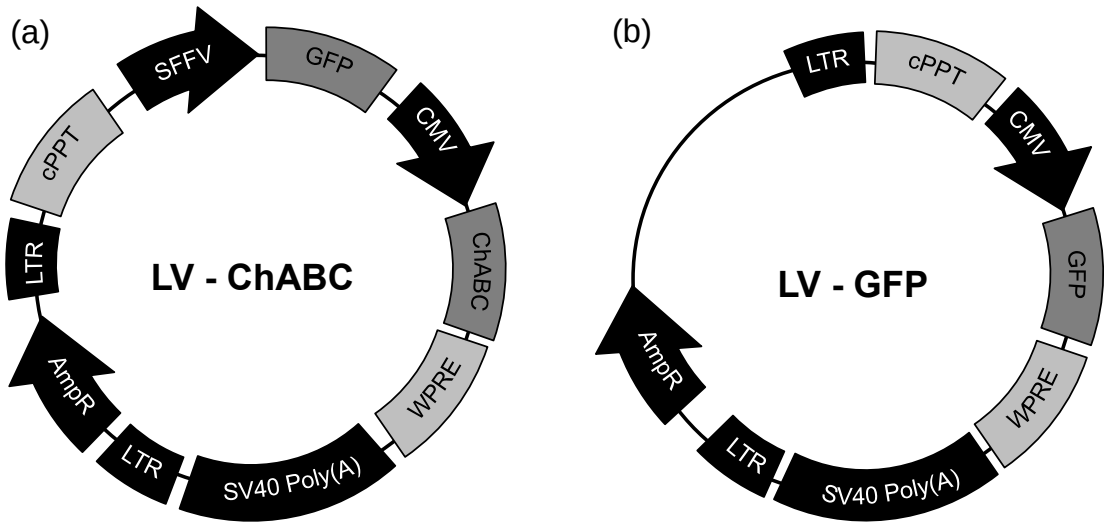


Figure 1. Schematic drawings of the transfer plasmids containing *chondroitinase ABC* and *green fluorescent protein* (LV-ChABC) in (a), and only *green fluorescent protein* (LV-GFP) in (b), to be incorporated into lentiviral vectors. *GFP* is under the control of a SFFV promoter in (a) and under the control of a CMV promoter in (b). LV-ChABC contains the mammalian-modified *ChABC* gene under the control of a CMV promoter in (a). Both vectors contain the cis-acting WPRE and the cPPT to enhance transgene expression and transduction efficiency and both plasmids contain the AmpR to allow for selective growth in *Escherichia coli* bacteria.
AmpR, ampicillin resistance; *ChABC*, chondroitinase ABC; CMV, cytomegalovirus promoter; cPPT, central polypurine tract; *GFP*, Green fluorescent protein; LTR, long terminal repeat; SFFV, spleen focus forming virus promoter; SV40 Poly(A), simian virus 40 polyadenylation signal; WPRE, woodchuck hepatitis post-transcriptional regulatory element.

2.2 Cell cultures

Olfactory mucosa cells were collected from fresh canine cadavers within 10 minutes of euthanasia. These animals were companion dogs presented to our clinic and euthanized for medical reasons independent of this work; consent was obtained from the animals' owner and ethical permission was granted by our local ethical committee (Veterinary Investigation Number: 13/033). Cells were collected via endoscopic nasal mucosa biopsy and prepared according to previously published methods (49). Briefly, they were maintained in culture medium containing DMEM, 10% FBS, 2 µM forskolin and 20 ng/mL neuregulin-1. HEK293T cells were cultured in DMEM, 10% FBS, 2 mM L-Glutamine and 1 x MEM. HeLa cells were cultured in DMEM, 10% FBS and 2 mM L-Glutamine and Neu7 cells in DMEM and 10% FBS. All culture media was supplemented with 1% penicillin and streptomycin. All cells in culture were maintained by replacing half of the media with fresh media every 3-4 days.

2.3 Cell transduction

For transduction experiments, OMCs, HEK293T cells and HeLa cells were passaged into 24 well plates (1.9 cm^2 diameter wells) at 40 x 10^3 cells/cm^2. Replicates of at least 3 wells per experimental condition were performed. Cells were transduced with the lentiviral vector containing *pRRL-CMV-ChABC-SFFV–GFP* (LV-ChABC) at varying multiplicities of infection (MOI) from 1 to 20 or *pRRL-CMV-GFP* (LV-GFP) at MOI 1, to act as a negative control for ChABC production. Viral vector was added at 1 day *in vitro* and cell supernatant samples were collected 7 days following transduction. Replicate OMC cultures were grown in 4 well plates on glass coverslips for immunocytochemistry. For Western blot samples CSPG rich media was added to cell cultures 24 hours prior to collection. The CSPG rich media was obtained from Neu7 cell culture media as previously described by Muir *et al* (27).

2.4 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 minutes and immunolabeled using mouse anti-nerve growth factor receptor ($p75^{NGF}$) (MAB5264, Millipore, Germany; 1:200), rabbit anti-fibronectin (A0245, Dako, Denmark; 1:400) and chicken anti-GFP (Abcam; 1:2000). Secondary antibodies were anti-mouse 546 (Abcam, UK; 1:500), anti-rabbit 660 (Abcam, UK; 1:400) and anti-chicken 488 (Abcam, UK; 1:500). Coverslips were mounted using hard-set mounting medium containing DAPI (Vectashield). The transduction efficiency at varying MOI was calculated using ImageJ by counting the number of $p75^{NGF}$ positive cells (OECs) that were also GFP positive for each MOI. Immunocytochemistry was not performed on HEK293T or HeLa cell cultures as they were used primarily to measure ChABC production from differing cell lines.

2.5 Western blots

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. Briefly, samples were combined with 5 x non-reducing Laemmli sample buffer (1:4), boiled for 2 minutes, separated by SDS-PAGE (5% acrylamide gel) and wet transferred onto a nitrocellulose membrane prior to Western blotting. Samples of concentrated cell culture supernatants were used to detect intact CSPGs or their digestion products. CSPG rich media incubated with commercial ChABC (C3667, Sigma-Aldrich, UK; 20 mU) for 3 hours at 37°C was used as a positive control for CSPG digestion. LV-GFP media was used as a negative control. Primary antibodies were mouse anti-NG2 (sc33666, Santa Cruz, Texas; 1:1000), mouse anti-C4S (63651, MP Biomedicals, California; 1:250) and rabbit anti-chondroitinase ABC polyclonal anti-peptide antibody (from Dr. Elizabeth Muir, University of Cambridge). Membranes were blocked in 5% semi-skimmed milk for 1 hour at room temperature and then incubated in primary antibody, diluted in blocking solution, overnight at 4°C. Membranes were washed with tris-buffered saline with tween (TBS-T) prior to incubating in secondary antibody for 1 hour at room temperature. Secondary antibody was either horseradish peroxidase linked anti-mouse IgG (NA931, GE Healthcare, UK; 1:10,000) or anti-rabbit IgG (NA934, GE Healthcare, UK; 1:10,000). Membranes were then washed in TBS-T before applying Amersham ECL prime Western blotting detection reagent (RPN2232,

GE Healthcare, UK). Blots were visualised on Amersham ECL hyperfilm (28906837, GE Healthcare, UK).

2.6 In vitro Morgan-Elson enzyme assay

This assay (50) detects the breakdown products of CSPG digestion. N-acetylation of the disaccharide products cleaved from CSPGs when exposed to active ChABC enzyme results in a colored product. The assay was more recently adapted to allow a quantitative measure of active ChABC enzyme concentration using a spectrophotometer to quantify the resultant color change (27). The reaction mixture consists of 100 µL of 40 mM sodium acetate, 40 mM Tris-Cl (pH=8.0) and 10 mg/mL chondroitin-6-sulphate (substrate) mixed with 50 µL of sample (*i.e.* the cell supernatant). N-acetyl-galactosamine was added to one tube to act as a positive control. The reaction was incubated at 37°C for 20 minutes then boiled for 1 minute to stop the reaction. 100 µL of 0.8 M potassium tetraborate tetrahydrate solution was added and the mixture was boiled for 7 minutes before being cooled on ice. 1 mL of glacial acetic acid was added and samples were then centrifuged at 10,000 rpm for 33 minutes. The resultant supernatant was transferred to new 1.5 mL tubes and 0.4 mL of Morgan-Elson reagent (10 g paradimethylamino-benzaldehyde in 100 mL of glacial acetic acid containing 12.5% hydrochloric acid) was added and incubated at 37°C for 20 minutes. Absorbance was measured at 550 nm in a disposable cuvette. ChABC enzyme units per mL were calculated using a formula (Appendix EqA.1) that takes into account absorbance readings compared to blank (water) and the positive control (N-acetyl-galactosamine). With regard to the final enzyme concentration, one unit will liberate 1.0 µmol of 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-ene-pyranosyluronic acid)-6-O-sulfo-D-galactose from chondroitin-6-sulfate per minute at pH=8.0 at 37°C. Morgan-Elson assays for each experiment were run in a single day using the same solutions across all samples. All results are expressed as mean (standard deviation).

3. Results

3.1 Phenotypic examination of transduced canine olfactory mucosa cultures

In order to test whether lentiviral vector mediated gene transfer or transgene expression caused changes in OMC characteristics, cultures of OMCs were phenotypically examined under high magnification for cell morphology and the expression of cell markers classically used for OEC identification (i.e. $p75^{NGF}$) and OMC culture contaminants (i.e. fibronectin). Based on the terminology proposed by Li *et al* (42) these fibronectin positive cells are known as olfactory nerve fibroblasts. There was no change in cell morphology or cell marker expression when comparing control cell cultures with virally transduced cultures (Figure 2a, 2b and 2d, 2e respectively). Virally transduced cells can be identified by the expression of GFP (Figure 2d, 2f). Both cultures showed $p75^{NGF}$ positive cells with a bipolar and elongated morphology (Figure 2b, 2e and 2f) as previously described (44, 49, 51). In contrast, the fibronectin positive olfactory nerve fibroblasts, often present in olfactory mucosa cultures (52) had a flat, rounded appearance (Figure 2c and 2g) and on average represented 12% (SD = 3%) of the cells in all cultures. Quantification of the proportion of $p75^{NGF}$ positive cells in olfactory mucosa cultures is presented in Figure 3c.

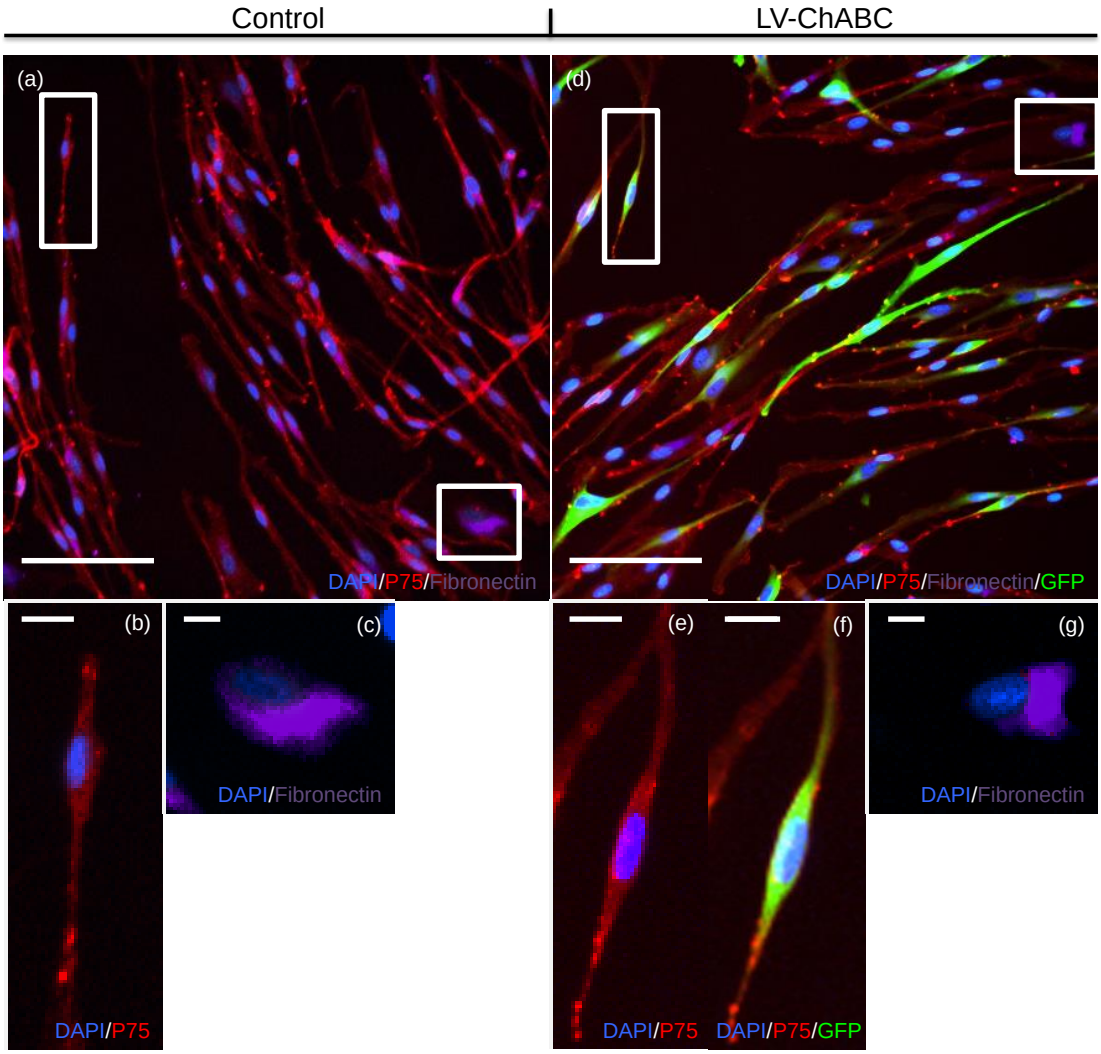


Figure 2. The morphology of cells in canine olfactory mucosa cultures and $p75^{NGF}$ marker expression was unaffected by lentiviral vector transduction with LV-ChABC. Higher magnification images of highlighted regions from (a) and (d) are represented below each low magnification image. Panels a, b and c are from control cultures whereas panels d, e, f and g are from transduced cultures. Lentivirally transduced $p75^{NGF}$ cells (i.e. olfactory ensheathing cells - OECs) display a typical bipolar morphology and $p75^{NGF}$ expression (d, e, f) comparable to control olfactory mucosa cultures not exposed to virus (a, b). Olfactory nerve fibroblasts, making up a mean of 12% of olfactory mucosa cultures, display a more rounded morphology and express fibronectin but not $p75^{NGF}$ (c, g). Virally transduced cells express the reporter protein GFP (d, f) whereas non-transduced cells do not (a, b). DAPI staining represents cell nuclei. Scale bar in (a) and (d) is 100 µm and 10 µm in (b, c, e, f and g).

3.2 Lentiviral transduction and purity of canine olfactory mucosa cultures

Two lentiviral vectors were used to transduce HEK293T, HeLa and OMCs in culture (Figure 1). The LV-GFP construct was used as a negative control as it does not contain the *ChABC* transgene. The LV-ChABC construct contains the *ChABC* transgene along with the *GFP* reporter gene. OMCs produced GFP following lentiviral transduction, allowing quantification of the transduction efficiency of the viral vector (Figure 3a and

3b). At MOI 1 the percentage transduction of LV-ChABC was very low. With increasing MOI the percentage transduction reached 34% (SD = 2%) at MOI 10. At MOI 1 the percentage transduction of LV-GFP was similar to that seen in LV-ChABC at MOI 5. The cell counts per microscopic field, used to calculate the cell culture purity, did not decrease with increasing MOI and cells continued to expand, suggesting cell survival was unaffected by viral transduction.

Olfactory mucosa cultures contained >85% $p75^{NGF}$ positive cells (Figure 3c). This was determined based on the proportion of $p75^{NGF}$ positive cells identified from immunocytochemistry of OMC cultures divided by the total number of DAPI positive cells per field. The remaining cells were likely to be contaminating olfactory nerve fibroblasts often present in olfactory mucosa cultures (Figure 2). Increasing MOI of lentiviral transduction did not significantly affect cell culture purity (Figure 3c) and the number of $p75^{NGF}$ positive cells contributing to this data did not vary with increasing MOI.

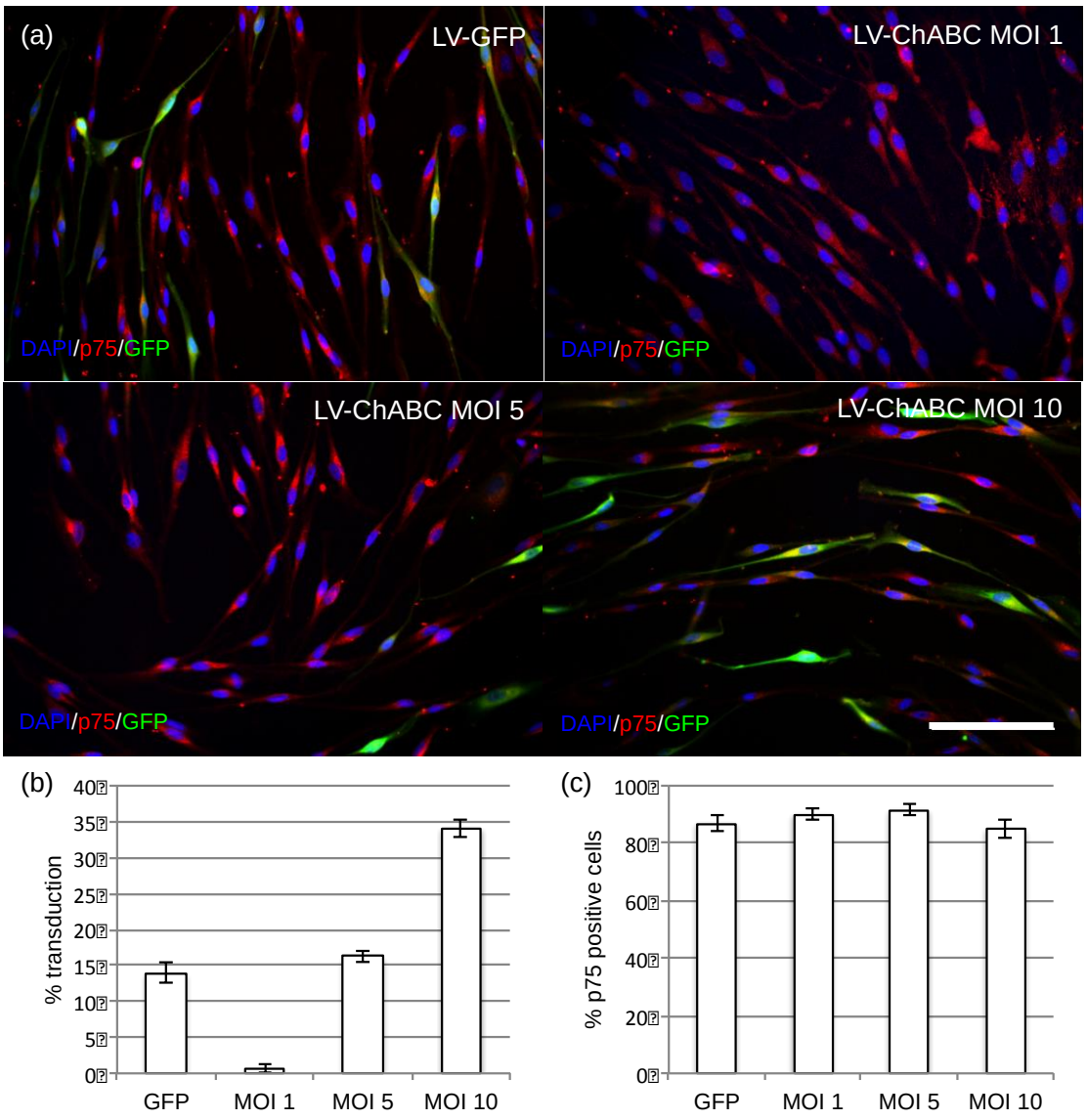


Figure 3. Transduction efficiency using LV-GFP or LV-ChABC and the purity of canine olfactory mucosa cultures. (a) Representative immunofluorescent images of canine OMC cultures stained with $p75^{NGF}$ and GFP following transduction with LV-GFP (top

left image) and or with LV-ChABC at multiplicity of infection (MOI) 1, 5 and 10; there is an increasing expression of the reporter protein GFP; DAPI staining represents cell nuclei in blue - scale bar, 100 µm; (b) Graph representing the percentage transduction of canine OMCs expressing $p75^{NGF}$ with LV-GFP and with varying MOI of LV-ChABC. Percentage transduction reached 34% with MOI 10; (c) Graph representing the percentage of $p75^{NGF}$ positive cells in OMC cultures out of the total number of cells in culture; OMC cultures contained >85% $p75^{NGF}$ positive cells and this percentage remained relatively constant with varying MOI.

3.3 Production of active ChABC from genetically modified canine olfactory mucosa cultures

Functional ChABC was detectable in cell supernatants of LV-ChABC transduced cell cultures (Figure 4). Enzyme concentrations increased with increasing MOI. This correlates with the increasing percentage transduction of cells at increasing MOI as shown in Figure 3c. Enzyme units were determined using the Morgan-Elson assay that detects active enzyme by detecting a breakdown product of CSPG digestion. At MOI 10, ChABC concentrations reached 0.09 (SD = 0.01) U/mL in canine OMCs (Figure 4a). The ChABC enzyme production for the GFP negative control cells was recorded as 0.004 (SD = 0.00) U/mL, not zero, due to a small degree of error in the Morgan-Elson assay, and is highly unlikely to be due to the presence of ChABC given the make up of the vector construct. Levels of functional ChABC also increased with increasing MOI in lentivirally transduced HEK293T cell (Figure 4b) and HeLa cell (Figure 4c) supernatants. In HEK293T cell cultures, ChABC concentrations rose to 0.12 (SD = 0.01) U/mL at MOI 10, higher than the concentration of 0.09 (SD = 0.01) U/mL found using canine OMCs. There was a greater than 2 fold difference in ChABC concentrations between HEK293T and HeLa cell cultures with concentrations of 0.12 (SD = 0.01) U/mL in HEK293T cells at MOI 10 (Figure 4b) and 0.05 (SD = 0.01) U/mL in HeLa cells at MOI 10 (Figure 4c).

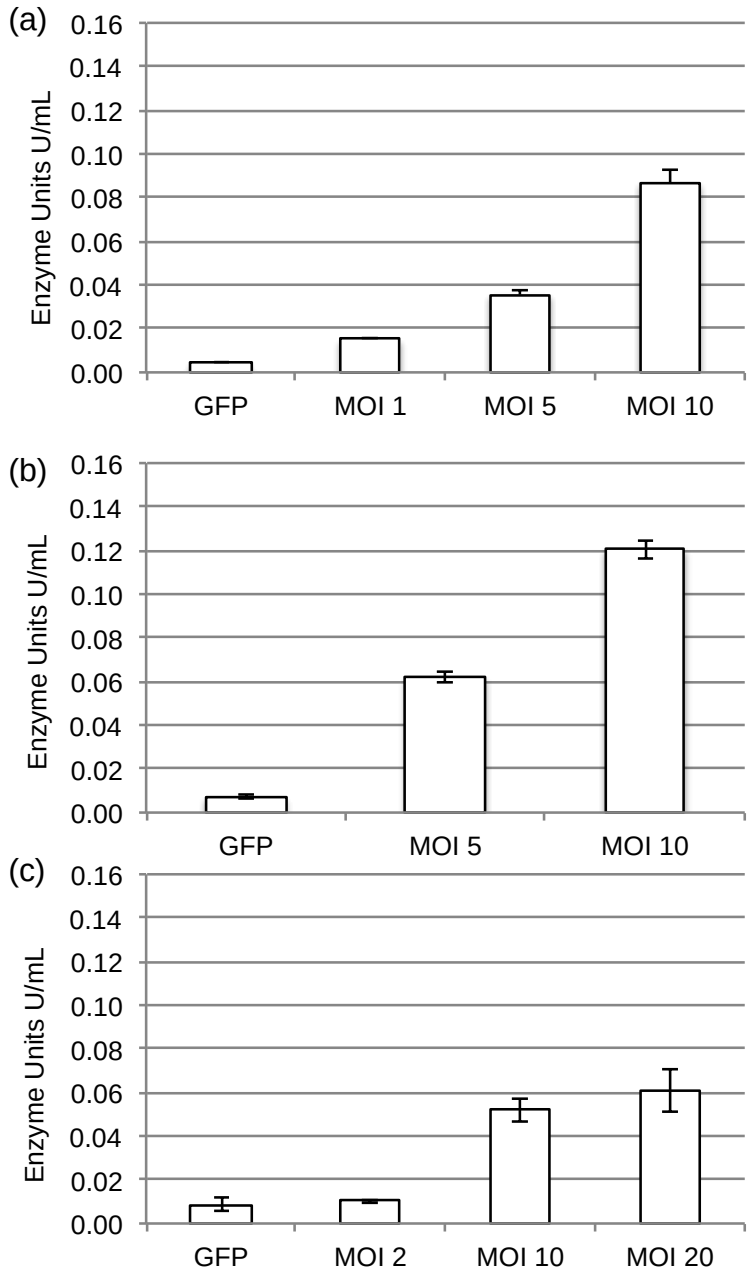


Figure 4. Chondroitinase ABC concentration measured in cell culture supernatant from genetically modified OMCs in (a), from HEK239T cells in (b) and from HeLa cells in (c). LV-GFP transduced cells were used as negative controls as the vector does not contain the *ChABC* transgene.

3.4 ChABC produced by canine olfactory mucosa cultures digest CSPGs

To further demonstrate that functional ChABC was produced by canine OMCs, we performed Western blotting to detect CSPGs, their digestion products and ChABC (Figure 5). Conditioned media containing CSPGs from Neu7 cells was incubated with transduced OMCs for 24 hours. As a positive control (+), media containing CSPGs was incubated with commercial ChABC for 3 hours at 37°C (Figure 5a and b, lane 1). The negative control (-) was the same media without exposure to ChABC (Figure 5a

and b, lane 2). In supernatant from LV-ChABC transduced canine OMCs, a core protein band was detected by NG2 immunolabelling (Figure 5a, lane 3), whereas conditioned media incubated in the presence of LV-GFP transduced cells demonstrated the characteristic smear of the intact CSPG, NG2 (Figure 5a, lane 4). Conversely, conditioned media incubated with OMCs incorporating LV-ChABC, demonstrated the presence of the C4S CSPG stub region that is only exposed following ChABC digestion (Figure 5b, lane 3). As expected, no C4S stubs were detected in conditioned media incubated with OMCs incorporating LV-GFP (Figure 5b, lane 4). ChABC was detected in supernatant from LV-ChABC transduced OMCs but absent from LV-GFP transduced OMCs (Figure 5c).

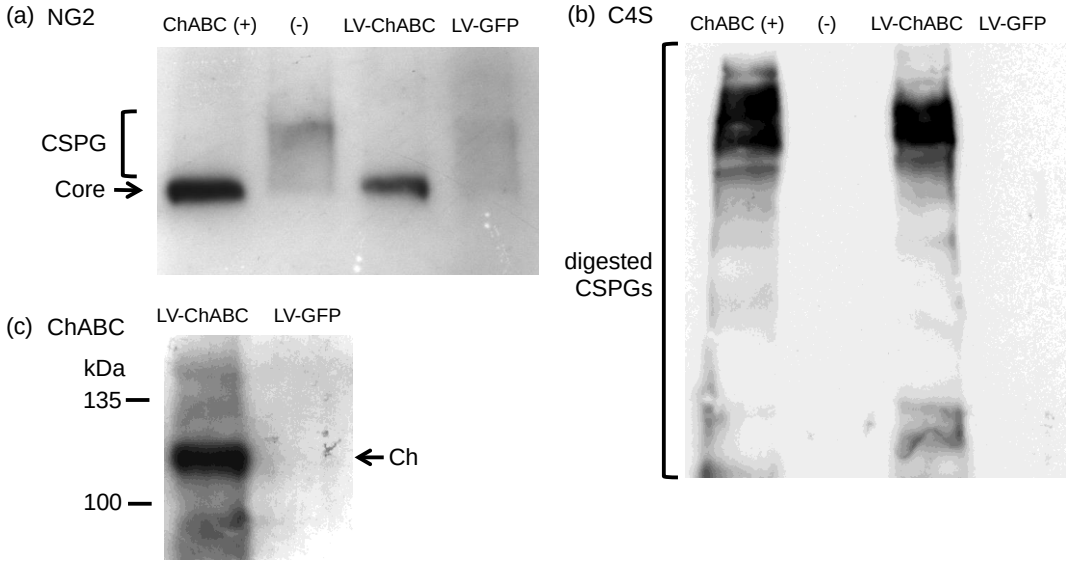


Figure 5. Enzymatically active mammalian chondroitinase ABC (ChABC) is secreted from genetically modified canine OMCs. CSPG rich media was added to cell supernatants 24 hours prior to sampling for Western blots, and labelled with antibodies to: (a) the CSPG NG2; (b) C4S, the carbohydrate stub revealed by ChABC activity; and (c) ChABC. The first two lanes in (a) and (b) are positive and negative controls respectively. Commercial ChABC was incubated with CSPG rich media for the positive control (+) whereas CSPG rich media alone was used for the negative control (-). Olfactory mucosa cultures were transduced with either LV-ChABC or LV-GFP seven days prior to sampling. (a) NG2: this appears as a characteristic smear due to the glycosaminoglycan (GAG) side-chains in undigested CSPGs (lane 2). This is converted to a core protein by digestion with commercial ChABC (lane 1). OMCs incorporating LV-ChABC convert the CSPG NG2 from a characteristic smear to core protein, demonstrating digestion of the GAG side-chains (lane 3). As expected, there is no change in the characteristic smear with media from OMCs transduced with LV-GFP. (b) C4S: this labels CSPG stubs only revealed by ChABC digestion (lane 1). No immunoreactivity is seen with the negative control (lane 2). OMCs incorporating LV-ChABC digest CSPG revealing the C4S stub (lane 3) whereas LV-GFP cells demonstrate no ChABC activity (lane 4). (c) ChABC: non-glycosylated ChABC produces a band at around 110 kDa. This was seen in cell supernatant of LV-ChABC OMCs (lane 1) but not LV-GFP OMCs (lane 2).

4. Discussion

In this study, we have shown that canine OMCs can be genetically modified to produce a functional ChABC enzyme. We chose to use cells of canine origin collected from the olfactory mucosa to allow further development of a genetically modified cell transplant for testing in the canine model of SCI (44, 53, 54). Dogs suffer a high rate of thoraco-lumbar SCI caused by mixed contusive-compressive disc herniation, similar to thoracic injuries sustained by humans. Using dogs with SCI is advantageous because their injuries recapitulate the clinical characteristics of SCI seen in human patients, including their heterogeneity, extent and complexity. Therefore, detection of functional improvement in the canine model is likely to translate into meaningful clinical benefits in people with SCI.

The collection of olfactory mucosa was done using a minimally invasive technique using nasal endoscopy, a methodology that can be repeated in human patients and represents a low risk procedure for cell collection (55, 56). It allows collection of cells from individual patients for autologous transplantation, overcoming the problem of possible immune rejection. Ruitenberg et al (57) purified their OEC cultures, using a technique that multiple other groups have performed (39, 42, 58, 59). Using our culture methods for canine OMCs, adapted from Jeffery et al (54), we achieved $p75^{NGF}$ positive OEC purities of >85% (Figure 3c). We chose not to purify these cultures, with the aim of eliminating olfactory nerve fibroblasts, as research has shown that a combination of OECs and olfactory nerve fibroblasts may be required to see the positive effects of these cell transplants (44, 60, 61).

Significant quantities of functional ChABC were detectable in cell supernatants of monolayers of lentivirally transduced OMCs without the need to concentrate samples. A previous study producing chondroitinase AC (from *Pedobacter heparinus*) using an adenoviral vector required cell supernatants to be concentrated for detection of enzyme (62). However, the absolute concentrations of ChABC produced are difficult to compare to previous *in vitro* data (27, 28, 62, 63) due to variations in experimental protocols and enzyme detection methods.

Multiple strategies have been developed to remove the axon inhibitory properties of CSPGs at the site of SCI (for a review, see 64). Degradation of CSPG side-chains using ChABC has been established as one approach for over a decade (11). As previously discussed, repeated delivery of enzyme, to overcome the problem of rapid loss of activity at body temperature, has hindered the translational potential of this therapy. One group has taken the approach of thermo-stabilizing ChABC and achieved sustained delivery of the enzyme for up to 6 weeks using a hydrogel-microtube scaffold (65). Other groups have pursued gene therapy as a means to improve sustained enzyme delivery (22, 23, 28, 29, 62, 63). An alternative approach to disabling the inhibitory CSPGs is to target the CSPG receptor present on neurons: protein tyrosine phosphatase σ (PTPσ) (66). Competitive inhibition of the receptor for PTPσ using a peptide mimetic of PTPσ has been shown to improve urinary function in rats (67). Effective knockout of PTPσ using lentiviral delivery of small interfering RNAs also led to motor and sensory recovery along with neurite outgrowth (68). However, it has also been shown that the products of CSPG enzymatic digestion encourage neurite outgrowth and have an immunomodulatory role in inflammatory central

nervous system lesions (69, 70). Therefore, targeting a specific CSPG receptor such as PTPσ, may not produce all the beneficial effects of ChABC.

The combination of OECs and ChABC (plus a Schwann cell graft) has already been tested in a complete spinal cord transection model where the addition of ChABC improved both bladder and motor function (45, 46). The feasibility of genetically modifying OECs from the olfactory bulb for SCI was previously demonstrated by Ruitenberg et al (57). OECs expressing GFP using a lentiviral vector were transplanted into the injured rat spinal cord and transgene expression persisted in transplanted OECs for at least 4 months. If the *ChABC* transgene could be expressed for a similar length of time this is likely to be an adequate timeframe to promote axonal regeneration and plasticity in chronic SCI. Indeed, the same mammalian-modified ChABC transgene presented here has previously been shown to produce active ChABC for 2 months following injection into the injured spinal cord (23) and 3 months following lentiviral transduction of a Schwann cell transplant (28). More recently Reginensi, Carulla (71) genetically modified OECs from the bulb to produce the Nogo receptor ectodomain (NgR ecto). This NgR ecto binds to myelin-associated inhibitors preventing their binding to the NgR complex present on axons and removing the inhibitory properties of myelin breakdown products following SCI. NgR ecto also increased OEC migration *in vitro* and *in vivo,* a mechanism thought to be key to their function (72). Increased migration was also found over CSPG substrates with the addition of ChABC. In future studies, we will examine the migratory properties of our ChABC-expressing OECs.

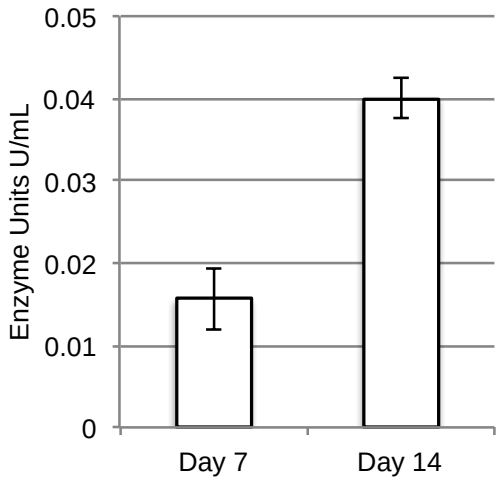
The plasmid construct presented here (Figure 1a) is capable of producing both GFP and ChABC via different internal promoters, SFFV and CMV respectively. It was noted that the intensity of GFP as a result of the LV-GFP construct (Figure 1b), where GFP is under the influence of a CMV promoter, was significantly higher than the GFP produced by the LV-ChABC construct (Figure 4). This is likely due to the efficacy of the different promoters. This may have artificially elevated the transduction percentage of the LV-GFP construct at MOI 1 as cells were more easily identified during microscopy and cell counting. This may also indicate that the transduction percentage of the LV-ChABC construct could be higher than presented if a more powerful promoter was used for the expression of GFP. The co-expression of GFP using these constructs will aid tracking of cell transplants in experimental models, providing more accurate information on transplant survival, location and migration.

Previous studies have used a MOI of up to 1000 in adult rat OECs to achieve transduction percentages close to 100% whereas parallel experiments in HEK293T cells only required a MOI of just 25 (57). We refrained from using such high MOI in our OEC cultures as even at our relatively low transduction rates sufficient ChABC was secreted. This was comparable to concentrations that have produced significant functional improvements *in vivo* in rat SCI models (11, 16). Despite the robust levels of ChABC seen *in vitro,* it is possible that a proportion of OMCs will not survive following SCI transplantation (73). Therefore, with our current transduction efficiency of 34%, there may not be enough surviving ChABC-producing cells at the site of injury to achieve the desired *in vivo* efficacy. This will be addressed in future experiments by documenting the degree of CSPG digestion *in vivo* following transplantation of this genetically modified cell type. However, if necessary we can increase the transduction percentage of the OMCs by using fluorescence-activated cell sorting to select OMCs expressing GFP (74, 75), and therefore ChABC, leading to a cell transplant with closer

to 100% transduction. It should be noted that from a clinical standpoint, OMC transplants are more likely to be given in the 'chronic' phase of SCI, at which point cell survival is greater than with acute transplantation (73). Clinical transplantation protocols will also include injection of cells cranial and caudal to the lesion, allowing cells to migrate into the lesion (76), which could also improve efficacy. In preliminary work we have seen active ChABC produced for the 2-week period of cell culture following transduction and the levels of enzyme produced tend to increase over time (see Supplementary Figure 1). In future experiments we will assess ChABC production over longer time frames *in vitro* and by transplanted OMCs *in vivo*. We anticipate that OMCs surviving transplantation will stop dividing to ensheath and re-myelinate axons.

5. Conclusion

We have provided here a method to genetically modify OECs to secrete active ChABC. In addition, co-expression of GFP will aid future *in vivo* work using this engineered cell type. Transplantation of OECs holds great promises for spinal cord repair because their efficacy to restore locomotion has been shown in several models including the canine natural model of SCI, although they do not seem to help recovery of brain controlled functions. We propose that combining OECs with ChABC, as we have done here, might further enhance the potential of OEC transplants to restore these complex neurological functions.



Supplementary Figure 1. Chondroitinase ABC concentration in cell culture supernatants from a separate line of canine genetically modified OMCs kept in culture for 14 days. The initial cell numbers used for these experiments were lower than those used in Figure 4 in order to avoid cells reaching confluence within 7 days, hence the lower overall ChABC concentrations.

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Appendix

$$\text{Enzyme Unit (U/mL)} = \frac{A585}{2.38} \times \frac{0.1}{G} \times \frac{1}{10} \times \frac{1}{E}$$

EqA.1 - Mathematical formula for determining ChABC enzyme concentration following Morgan-Elson assay. Abbreviations: A585, sample absorbance minus blank absorbance; G, standard absorbance minus blank absorbance; E, volume of enzyme solution.

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